

The influence of cadmium and zinc ions on the interferon and tumor necrosis factor production in bovine aorta endothelial cells

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Abstract

The influence of CdCl_2 , used at 1, 10 and 100 μM concentration, and ZnCl_2 at 1, 10 and 100 μM concentration on the production of interferon (IFN) and tumor necrosis factor (TNF) in bovine aorta endothelial cells (BAECs) was examined. BAECs were treated with cadmium ions or zinc ions alone or together with cytokine inducers: Newcastle disease virus (NDV) and lipopolysaccharide (LPS). Cadmium ions at 1 and 10 μM concentration, used alone induced a low, but detectable TNF activity in BAECs, and zinc ions at 1, 10 and 100 μM concentration induced both IFN and TNF activity. In contrast to that, cadmium added to BAECs together with the virus or LPS as cytokine inducers significantly inhibited the production of IFN and TNF. Cadmium effect depended on the concentration used, and 1 and 10 μM CdCl_2 partially, but 100 μM cadmium completely inhibited the production of both cytokines. Zinc ions at 1 and 10 μM concentration, which only slightly inhibited the production of both cytokines, did not reconstitute cadmium-depressed IFN and TNF production. These data indicate that cadmium-induced depression of cytokine production in bovine endothelial cells, in response to viral and bacterial stimuli, cannot be reversed by zinc supplementation. © 2000 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Cadmium; Zinc; Endothelium; Interferon; Tumor necrosis factor

Abbreviations: Anti-BoIFN- α 1, antibodies against bovine interferon α 1; BAECs, bovine aorta endothelial cells; CPE, cytopathic effect; DMEM, Dulbecco's modified Eagle's minimal essential medium; EC, endothelial cells; FCS, foetal calf serum; IFN, interferon; MTT, tiazolyl blue; LPS, lipopolysaccharide; NDV, Newcastle disease virus; PBS, phosphate buffered saline; rHuTNF- α , recombinant human TNF- α ; rHuIFN, α -recombinant human IFN- α ; TCID₅₀, tissue culture infectious dose 50%; VSV, vesicular stomatitis virus.

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1. Introduction

Intoxication of animals with cadmium results in the reduction of their immunity to bacterial, viral and parasitic diseases. Several immune parameters such as the T and B lymphocyte number in lymphoid organs, phagocytic activity of peritoneal and spleen macrophages and the activity of the complement system was significantly reduced in animals treated with cadmium ions (Ciz et al., 1996; Boroskova and Dvoroznakova, 1997). It was found that natural killer cell (NK) functions were inhibited by the *in vitro* cadmium administration, but both inhibitory and stimulatory effects on NK cell function were found after *in vivo* cadmium treatment depending on the time of exposure (Stacey, 1986; Cifone et al., 1989, 1990; Yucesoy et al., 1997). A high level of cadmium has also an immediate suppressive effect on the antibody response in animals, however, low cadmium levels were reported to enhance antibody synthesis (Borrel and Giardino, 1991). Cadmium can also influence the expression of genes related to inflammatory responses. The enhancement of transcription of the genes for cytokines IL-1 α , IL-1 β , IL-6, MIP-1, MIP-2 and TNF- α , as well as secretion of these proteins, was observed when CdCl₂ (2 mg/kg) was given orally to mice. On the other hand, *in vitro* 1 μ M CdCl₂ inhibited the activation and IL-1 β production in human monocytes treated with LPS (Beyersmann and Hechtenberg, 1997; Koropatnick and Zalups, 1997).

In contrast to cadmium, zinc is an important trace element both for the development and maintenance of the immune system. Zinc deficiency is associated with reduced resistance to several microorganisms, parasites, bacteria and viruses (Chandra and McBean, 1994; Cunningham-Rundles, 1996; Wellinghausen et al., 1997). It was found that 50–100 μ M zinc can induce cytokine production, predominantly IL-1, IL-6 and TNF- α in peripheral blood mononuclear cells (PBMC) and acts synergistically with LPS to induce cytokine production in leukocytes. At lower concentrations resembling its physiological level in serum (12–16 μ M), zinc augments proliferation as well as cytokine secretion in PHA-stimulated

leukocytes (Driessen et al., 1994, 1995a,b; Wellinghausen et al., 1996).

It has been well established that cadmium influences intracellular zinc homeostasis, but on the other hand zinc influences cadmium accumulation in cells. It has been determined that 50 μ M CdCl₂ inhibits zinc uptake by isolated renal proximal cells, but coincubation of renal epithelial cells with 30 μ M ZnCl₂ caused a significant reduction in Cd accumulation in cells. Moreover, both ions seem to use a common, saturable transport process in cells (Endo and Shaikh, 1993; Yoshida et al., 1993).

Endothelial cells have a potent role in inflammatory and immune reactions. Because of their strategic location, endothelial cells are able to interact with other cells in both the bloodstream and vessel wall. On exposure to various environmental stimuli they undergo profound changes in gene expression and function. Endothelial cells are influenced by cytokines produced by other cells and are themselves capable of producing a number of cytokines (Mantovani et al., 1997; Krishnaswamy et al., 1999).

Cadmium is very toxic for endothelial cells cultured *in vitro* and accumulates in these cells. Zinc protects them *in vitro* against cadmium cytotoxicity by a zinc-induced decrease in the intracellular cadmium accumulation and sequestration of cadmium by metallothioneins (Kaji et al., 1992; Mishima et al., 1995).

As zinc is known as an inducer and enhancer of cytokine production in blood leukocytes and cadmium has been described as inhibitor of LPS-induced cytokine production in monocytes *in vitro* but enhancer of cytokine production *in vivo*, it was of interest to examine the effect of cadmium and zinc used separately and both metal ions together on cytokine production induced by the virus or bacterial lipopolysaccharide (LPS) in endothelial cells, which are in the organism a very important source of several cytokines with pleiotropic activity, among other things, the interferon (IFN) and tumor necrosis factor (TNF; Knop, 1990; Rink and Kirchner, 1996). In experiments we used 1, 10 and 100 μ M of zinc, and 1, 10 and 100 μ M of cadmium.

2. Materials and methods

2.1. Cell cultures

Cultures of bovine aorta endothelial cells (BAECs) originated from vessels obtained from freshly slaughtered steers and heifers ($n = 5$). The vessels were clumped before dissection from the surrounding tissue, excised, rinsed with phosphate buffered saline (PBS) and filled with 0.25% trypsin. After 30 min of incubation at 37°C endothelial cell suspension was collected and centrifuged ($250 \times g$ for 10 min). The cell pellet was resuspended and subsequently cultured in Dulbecco's modified Eagle's medium (DMEM), containing 10% foetal bovine serum, 100 U/ml of penicillin, 100 µg/ml streptomycin. The cells were grown in tissue flasks in a CO₂ incubator (5% CO₂) at 37°C. In this study BAEC at passages 3–5 were used. Endothelial cell identity was verified by direct phase contrast microscopy (cobblestone morphology) and by indirect immunofluorescence microscopy after staining for factor VIII-related von Willebrand antigen (Dako, Glostrup, Denmark). Further culturing of cells was performed on 24-well plates (Costar Corp., Cambridge, MA, USA). Cells at a density of 5×10^5 were transferred to wells and grown to confluency. Before cytokine induction the medium was changed to a fresh one supplemented with 2% of foetal calf serum (FCS) and antibiotics.

2.2. Induction of cytokines

Zinc chloride and cadmium chloride (Merck, Darmstadt, Germany) were dissolved under sterile conditions in aqua ad injectabilia to a stock solution of 10 mM, diluted in DMEM without serum (1:2), sterile filtered, further diluted in DMEM with 2% of FCS and added to BAEC cultures at concentrations from 100 pM to 100 mM, incubated at 37°C for 96 h and the viability of cells was assessed by the trypan blue exclusion test. For further experiments a nontoxic 1 and 10 µM and subtoxic 100 µM concentration of zinc ions and subtoxic 1 µM and toxic 10 and 100 µM concentration of cadmium ions were chosen. Zinc and cadmium ions were added to BAECs in small

volumes (0.1 ml) leading to final concentration of 100, 10 or 1 µM. Immediately after treatment with metal ions, BAECs were induced for cytokine production by adding Newcastle disease virus (NDV) 5 tissue culture infectious dose 50% (TCID₅₀) per cell and incubated at 37°C for 24 h, or induced by LPS (O111:B4, Sigma, St Louis, MO, USA) 10 µg/ml and incubated for 4 h at 37°C. After the indicated time, samples of the culture media were collected and immediately examined for cytokine levels.

2.3. Assay for cytokines

WEHI 164-murine fibrosarcoma cells (ECACC No 8702251) were used to measure TNF activity by estimating the percentage of cytotoxicity according to the method of Allen et al. (1993). WEHI 164 cells were grown on a 96-well microtiter plate for 24 h, the medium was removed and replaced with 50 µl/well of fresh medium with actinomycin D (final concentration 0.5 µg/ml). Fifty microlitres of serial 3-fold dilutions of the samples examined and standard recombinant human TNF-α (rHuTNF-α; lot 07, activity 1×10^8 IU/mg of protein, received from the Department of Bioorganic Chemistry, Center for Molecular and Macromolecular Research, Polish Academy of Sciences, Sienkiewicza 112, 90-363 Łódź) were added in triplicates to the wells and incubated for 24 h. The cytotoxic effect of TNF was determined by using the tiazolyl blue (MTT) method (Mosmann, 1983). The reciprocal of the highest dilution causing destruction of cells in 50% and compared to the standard was defined as 1 unit of TNF.

For IFN assay a cytopathic effect (CPE) inhibition method was used with vesicular stomatitis virus (VSV) as challenge. IFN samples were serially diluted (0.3 log) in 96-microtiter plates in triplicates, and bovine embryonic fibroblasts (BF), a strain obtained by trypsinization of foetal skin, were added and allowed to form a confluent monolayer during 24 h of incubation at 37°C. VSC was added and the plates were incubated at 37°C until CPE in wells with VSV (virus control) was 100% (generally after 48 h). The reciprocal of sample dilution that protects 50% of cells against

VSV was scored and calibrated in relation to a laboratory standard: human recombinant IFN- α 88 (a generous gift of Erik Lundgren, Umea University, Umea, Sweden) titrated against International Standard 69/19.

IFN activity was identified by neutralization with rabbit antibodies against bovine interferon α 1 (anti-BoIFN- α ₁) polyclonal antibodies (Genzyme, Cambridge, MA, USA). IFN containing samples were incubated for 3 h at room temperature with equal volumes of antibodies diluted 1:10 together with control samples to which the medium was added instead of antibodies, and titrated as described above.

2.4. Statistics

Data were analysed using Student's *t*-test. Significance was reported as $p < 0.05$.

3. Results

Cultures of BAECs on 24-well plastic plates were cultivated for 96 h in the absence or presence of various CdCl₂ or ZnCl₂ concentrations and the number of viable cells was measured by trypan blue exclusion test (Fig. 1). For further experiments two nontoxic 1 and 10 μ M and one subtoxic 100 μ M concentrations of zinc ions and one subtoxic 1 μ M and two toxic 10 and 100 μ M concentrations of cadmium were chosen.

BAECs cultures were incubated for 4 h (Fig. 2A, Fig. 3A) or 24 h (Fig. 2B, Fig. 3B) with cadmium or zinc ions alone or with the mixture of both ions. Moreover, the part of BAECs cultures were also induced for IFN and TNF production with LPS (Fig. 4A, Fig. 5A) or NDV (Fig. 4B, Fig. 5B) in the absence or presence of cadmium, zinc or a mixture of both ions.

Zinc ions used alone induced IFN production as early as 4 h after treatment of BAECs with 1, 10 and 100 μ M ZnCl₂ (Fig. 2A), and the IFN titers increased after 24 h of incubation (Fig. 2B). Zinc at 10 μ M concentration was the most potent IFN inducer. Zinc-induced IFN activity was not neutralized by polyclonal antibodies against BoIFN- α 1, however, the activity was stable at pH

2 for 12 h at 4°C and completely destroyed by the treatment with 0.25% trypsin solution (data not shown), indicating that it was probably IFN- β . Titers of zinc-induced IFN activity were significantly depressed by cadmium ions and this effect depended on cadmium concentration used (Fig. 2A and B).

When TNF activity present in the BAECs culture medium was titrated, we detected that both metal ions used separately were able to induce TNF activity (Fig. 3A and B). TNF induction ability of both metal ions was concentration-dependent in the range of concentrations from 1 to 10 μ M. The titers of metal ions-induced TNF were lower than those induced by LPS or NDV, but detectable by the biological method used. This activity, titrated in WEHI-164 cells was sensitive to trypsin treatment, and partially destroyed by treatment at 56°C for 30 min (data not shown). The mixture of both metal ions did not induce any additional TNF activity.

Both classical IFN inducers LPS and NDV, which are well known to induce IFN production in blood cells of human and bovine origin induced also high levels of IFN in BAECs. When 1, 10 or 100 μ M zinc ions were added together with IFN inducers to BAECs, a slight zinc concentration-dependent decrease in IFN titers was detected (Fig. 4A and B). In contrast to zinc, even 1- μ M cadmium ions caused a very strong depression of IFN production induced by bacterial LPS and NDV. When BAECs were treated with a mixture of both metal ions and with LPS or NDV, the levels of IFN were rather comparable to those induced in the presence of cadmium ions. It means that zinc was not able to reverse the suppressive activity of cadmium ions.

Zinc ions at 1 or 10 μ M concentration added together with LPS or NDV caused a very slight inhibition of TNF production, while 100- μ M zinc inhibited it significantly. In contrast to zinc, 1- μ M cadmium ions suppressed significantly TNF production stimulated by LPS or NDV. The mixture of both metal ions also caused a very strong depression of the TNF production induced by both cytokine inducers and TNF titers were rather similar to that produced in the presence of cadmium alone (Fig. 5A and B).

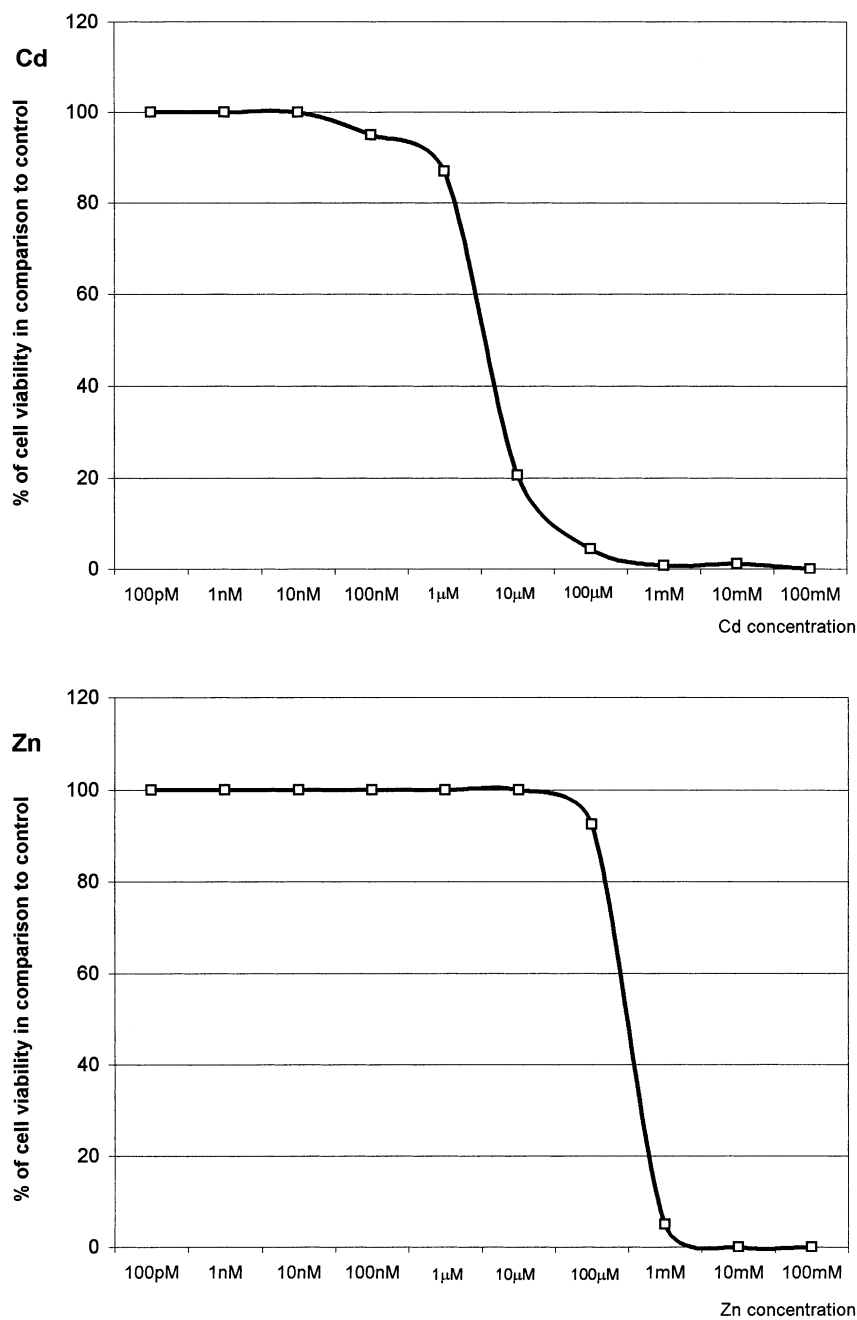


Fig. 1. Cadmium and zinc ions cytotoxicity in BAECs. Zinc chloride or cadmium chloride solutions were added to BAECs cultures at indicated concentrations and incubated for 96 h at 37°C. The viability of cells was assessed by the trypan blue exclusion test. Data are expressed as percentage of viability in comparison to control cells (not treated with metal ions).

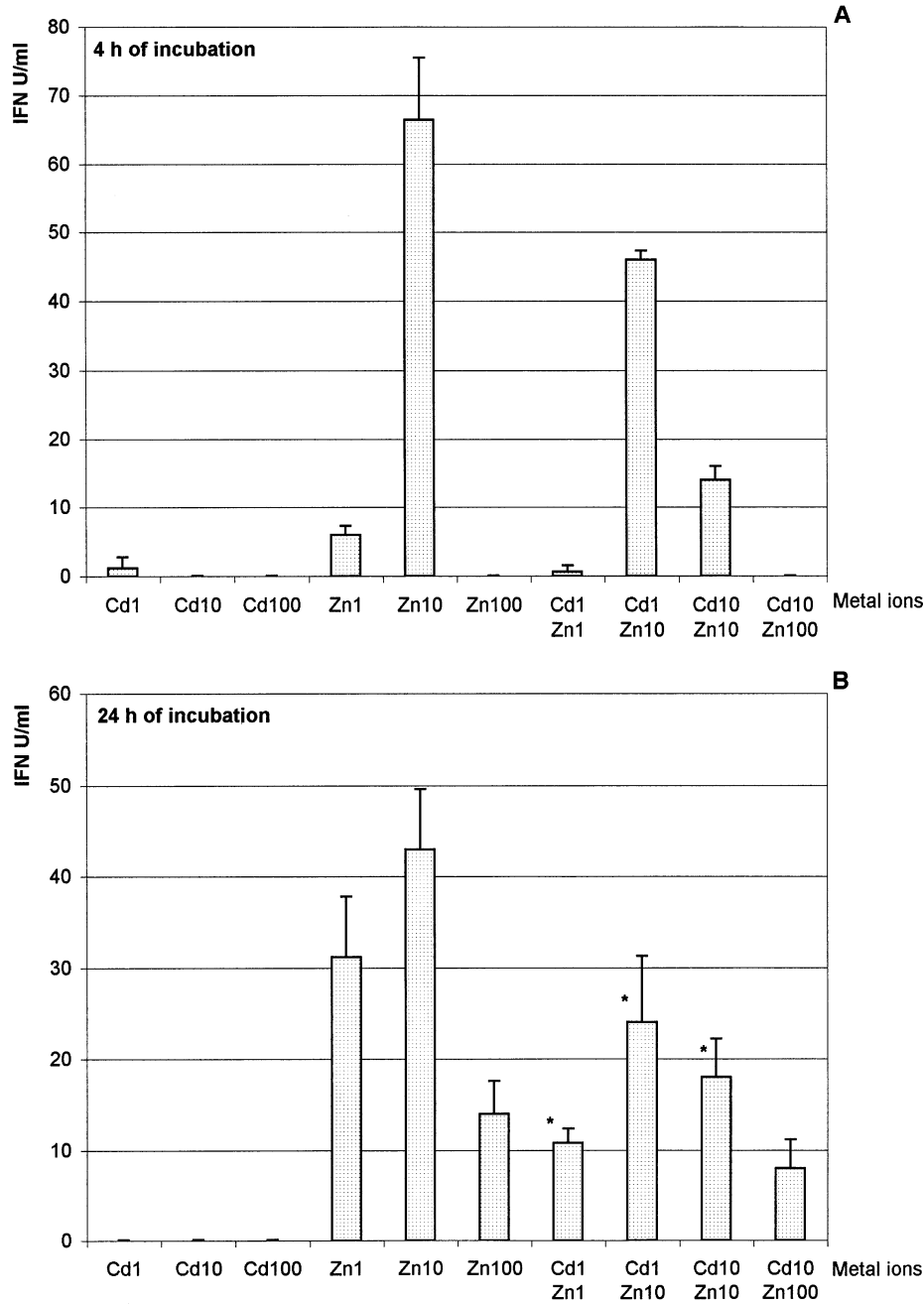


Fig. 2. Zinc and cadmium ions as IFN inducers. Zinc chloride or cadmium chloride solutions were added at indicated concentrations to BAECs cultures and incubated at 37°C for 4 or 24 h. Supernatants were collected and examined for IFN activity as described in Section 2. No IFN activity was detected in cell controls (not treated with metal ions).

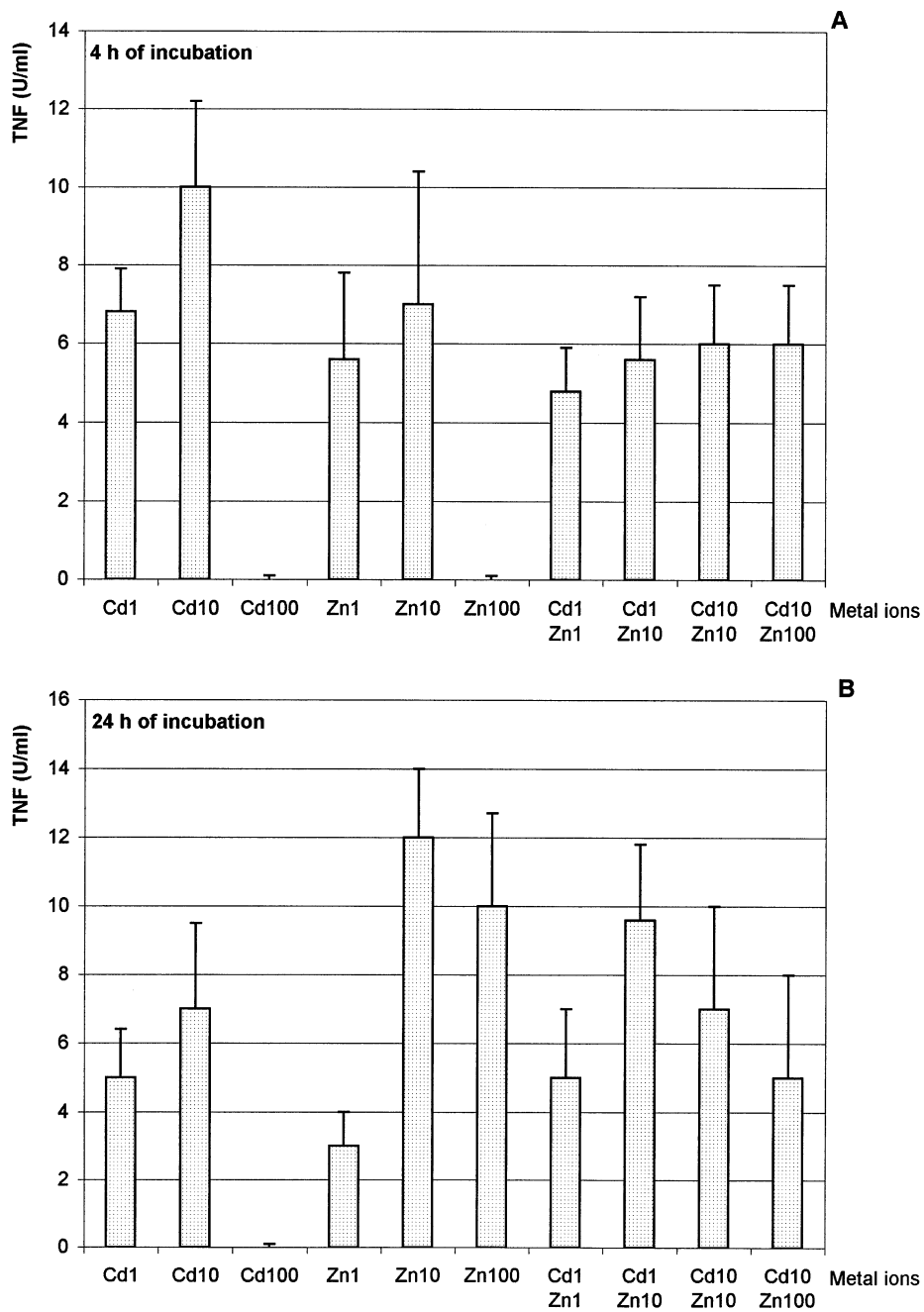


Fig. 3. Zinc and cadmium ions as TNF inducers. Zinc chloride or cadmium chloride solutions were added at indicated concentrations to BAECs cultures and incubated at 37°C for 4 or 24 h. Supernatants were collected and examined for TNF activity as described in Section 2. No TNF activity was detected in cell controls (not treated with metal ions).

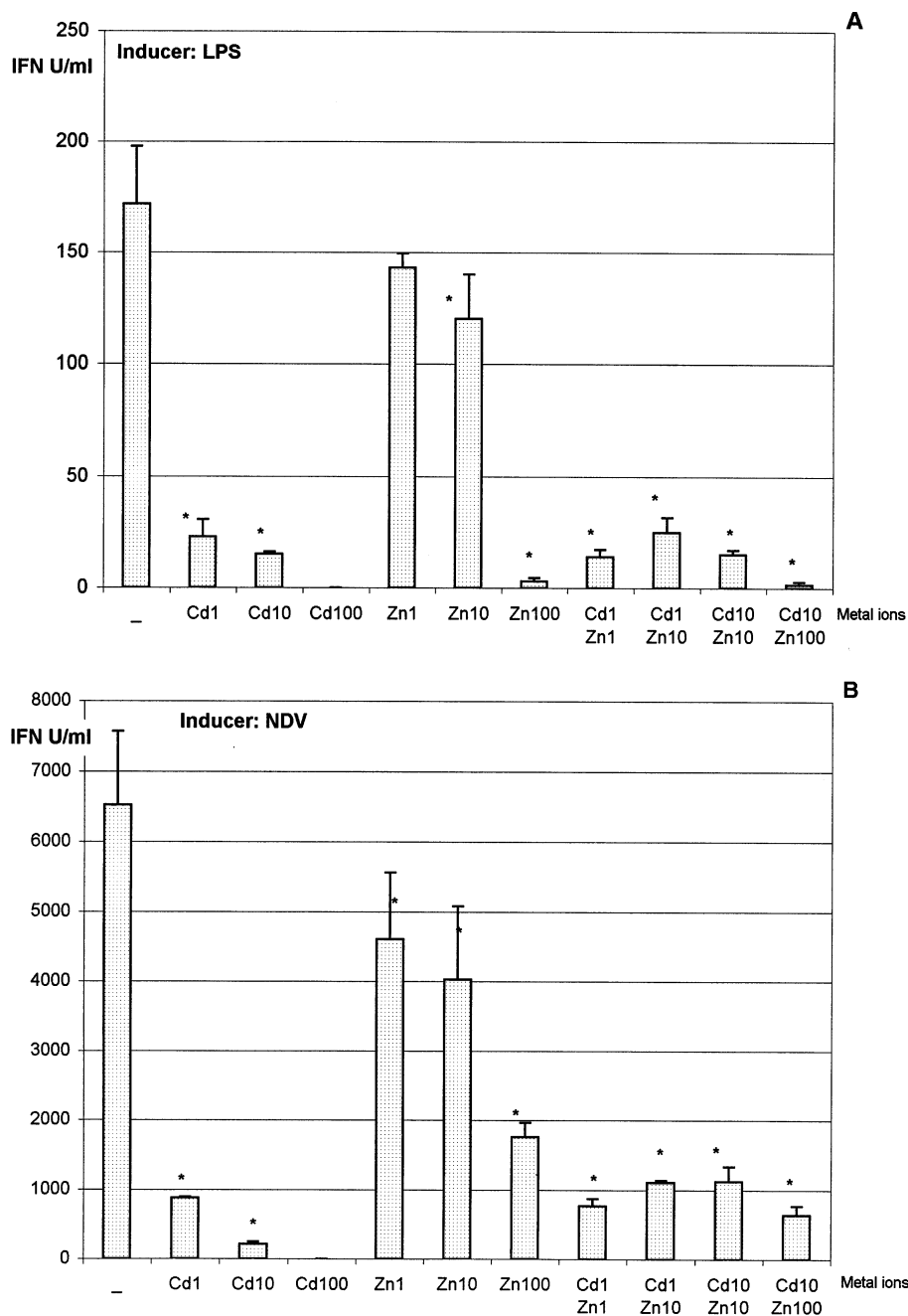


Fig. 4. The influence of cadmium and zinc ions on IFN production after induction by LPS (A) or NDV (B). BAECs were incubated with 1[D1], 10 or 100 μM CdCl_2 , with 1, 10 or 100 μM ZnCl_2 or with the mixture of both metal ions and additionally with cytokine inducers: LPS or NDV and incubated for 4 (LPS) or 24 h (NDV). The supernatants were collected and IFN titers were estimated as described in Section 2.

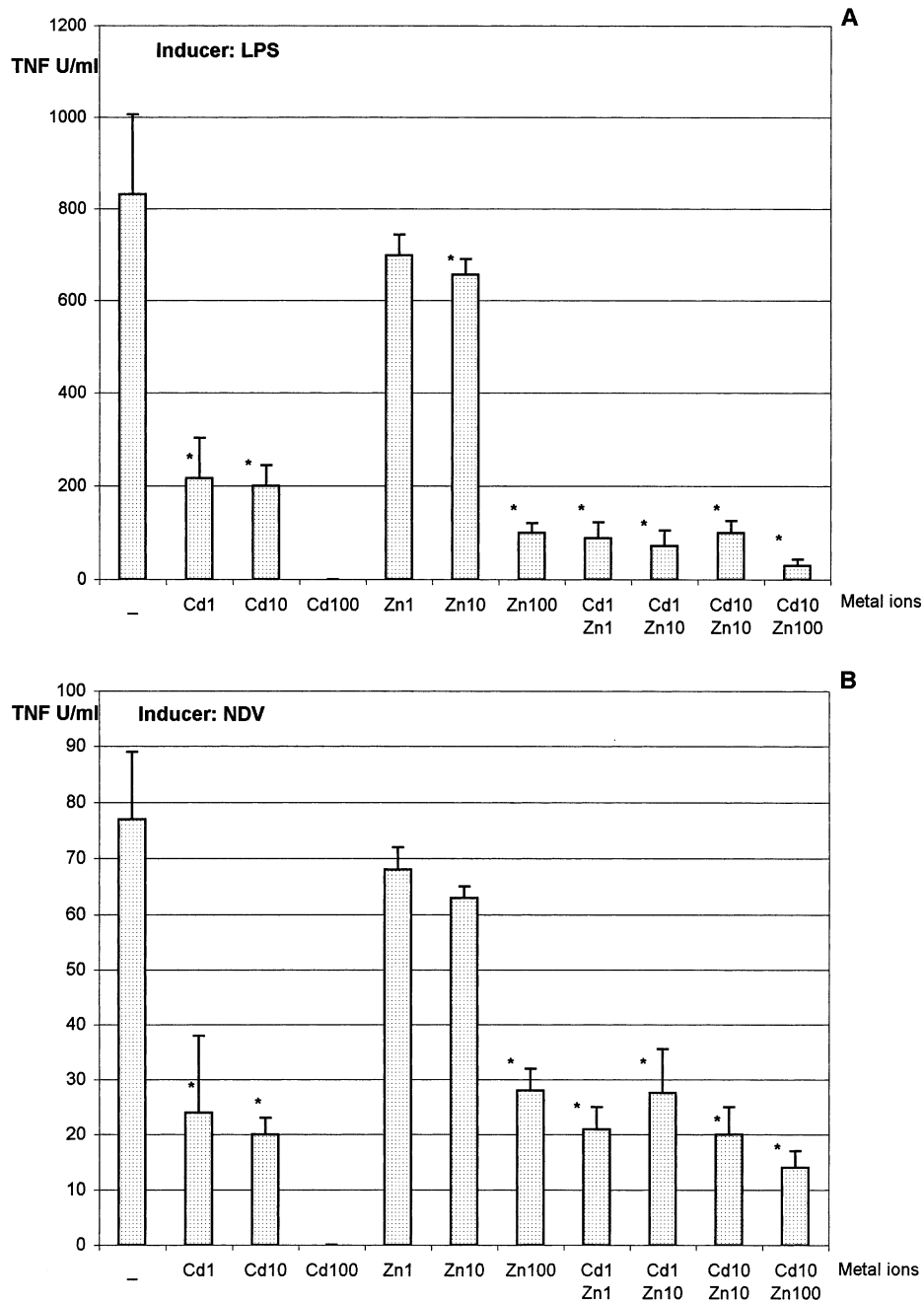


Fig. 5. The influence of cadmium and zinc ions on TNF production after induction by LPS (A) or NDV (B). BAECs were incubated with 1, 10 or 100 μM CdCl_2 , with 1, 10 or 100 μM ZnCl_2 or with the mixture of both metal ions and additionally with cytokine inducers: LPS or NDV and incubated for 4 (LPS) or 24 h (NDV). The supernatants were collected and TNF titers were estimated as described in Section 2.

4. Discussion

In our experiments, 1, 10 and 100 μM zinc supplementation induced IFN and TNF production in BAECs. Zinc at 10 μM concentration was the most potent IFN inducer. These results confirmed the results of other authors describing the stimulatory effect of zinc supplementation on some cytokine production, such as TNF- α , IL-1 β and IFN- α , in human PBMC (Driessen et al., 1994, 1995a,b; Wellinghausen et al., 1996). However, the concentrations of zinc ions active in cytokine stimulation in blood and endothelial cells differed significantly. In blood mononuclear cells zinc concentrations inducing cytokine production were several times higher (50–100 μM) than the physiological level of zinc ions (12–16 μM). Only when the blood of elderly persons which exhibited age-dependent defect in IFN- α and IL-6 production was examined, physiological zinc concentrations (15 μM) were able to reconstitute this defect (Cakman et al., 1997). In BAECs 100 μM zinc ions were partially toxic for cells (Fig. 1) and were less effective IFN inducer than 10 μM concentration (Fig. 2). As high zinc concentrations can inhibit T-cell proliferation and some activity of T cells (Wellinghausen et al., 1997), we can speculate that the concentrations of zinc required for cytokine induction in endothelial cells are rather similar to those required for optimally balanced T-cell function than those required by monocytes.

In our experiments we detected that TNF production can be induced not only by zinc but also by cadmium. This is not a new observation, because induction of IL-1, IL-6 and TNF- α in vivo in mice given cadmium orally or intraperitoneally has already been described (Beyersmann and Hechtenberg, 1997; Kataranovki et al., 1998), however, the tissue origin of these serum cytokines was not found. We suppose that, at least partially, cadmium-induced TNF activity was produced by endothelial cells.

We also detected that cadmium can inhibit IFN and TNF production induced by NDV and LPS. These observations are in agreement with those of other authors (Koropatnick and Zalups, 1997) who described a negative influence of cadmium on LPS-induced IL-1 β production in monocytes.

In several papers zinc-induced tolerance of endothelial cells to cadmium cytotoxicity was described (Kaji et al., 1992; Mishima et al., 1995) and this tolerance was postulated to be due to a decrease in intracellular cadmium accumulation and sequestration of cadmium by metallothioneins. Therefore, we expected that zinc supplementation could also reverse cadmium-induced inhibition of cytokine production after stimulation by LPS or the virus. Surprisingly zinc supplementation independently to its concentration used did not reverse cadmium-suppressed cytokine production.

We do not know the mechanism of this phenomenon, however, we suppose that the modulation by cadmium of TNF and IFN gene expression can be caused by its interference with cellular signalling at the level which cannot be reconstituted by zinc supplementation.

Dysregulation of cytokine production manifested as production without inflammatory inducer and also as a depression of cytokine production after induction with virus or bacterial product may be responsible to the reduction of immunity to bacterial and viral infections observed in cadmium exposed animals and humans (Thomas et al., 1985).

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